



TITLE:

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ROS are required for mouse spermatogonial stem cell self-renewal

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Running title: Involvement of ROS in spermatogonial self-renewal

Summary

Reactive oxygen species (ROS) generation is implicated in stem cell self-renewal in several tissues, but is thought to be detrimental for spermatogenesis, including spermatogonial stem cells (SSCs). Using cultured SSCs, we show that ROS are generated via AKT and MEK signaling pathways under conditions where growth factors GDNF and FGF2 drive SSC self-renewal, and instead stimulate self-renewal at physiological levels. SSCs depleted of ROS stopped proliferating, but they showed enhanced self-renewal when ROS levels were increased by addition of hydrogen peroxide, which induced phosphorylation of stress kinases p38 MAPK and JNK. Moreover, ROS depletion in vivo decreased SSC number in the testis, and NADPH oxidase1 (Nox1)-deficient SSCs exhibited reduced self-renewal division upon serial transplantation. These results suggest that ROS generated by Nox1 play critical roles in SSC self-renewal via activation of p38 MAPK and JNK pathways.

Introduction

Spermatogonial stem cells (SSCs) provide the foundation of spermatogenesis. Although small numbers of these cells exist in the testis, they undergo self-renewal division to generate spermatozoa throughout the life of male animals (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). Glial cell line-derived neurotrophic factor (GDNF) is a critical self-renewal factor for SSCs, and is secreted from Sertoli cells (Meng et al., 2000). Increased GDNF levels in GDNF transgenic mice causes the accumulation of undifferentiated spermatogonia and seminomatous tumor formation, whereas reduced GDNF levels in heterozygous knockout (KO) mice compromises SSC self-renewal and spermatogonial depletion. Together with fibroblast growth factor2 (FGF2) secreted from Sertoli cells (Mullaney and Skinner, 1992), SSC self-renewal can be recapitulated in vitro. Cultured SSCs, designated germline stem (GS) cells, continue to proliferate without losing SSC potential for > 2 years (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara et al., 2005). In vitro culture systems present a possibility for elucidating the mechanism of SSC self-renewal.

Ras was determined to be one of the critical regulators of SSC self-renewal. Ras is activated upon FGF2 or GDNF stimulation, and Ras inhibition by transfection of dominant-negative H-RasN17 was shown to abrogate GS cell proliferation and cause apoptosis. In contrast, transfection of activated H-RasV12 substituted for exogenous cytokines and induced cytokine-free GS cell proliferation, indicating that Ras is necessary and sufficient for SSC self-renewal (Lee et al., 2009). H-RasV12-transfected GS cells (Ras-GS cells) reconstituted spermatogenesis after

transplantation in the seminiferous tubules as well as produced seminomatous tumors, which were similar to GDNF-induced seminomatous tumors. Ras activity appears to be mediated in part by phosphoinositide 3-kinase (PI3K)-AKT and mitogen-activated protein kinase (MAPK) pathways. Both pathways are indispensable for SSC self-renewal because SSC self-renewal is suppressed by the addition of PI3K/AKT or MAPK/ERK1 kinase1 (MEK1) inhibitors in vitro (Lee et al., 2007; Oatley et al., 2007; Ishii et al., 2012). However, these molecules appear to play separate roles, because GS cells overexpressing AKT or MEK1 proliferate when supplemented only with FGF2 or GDNF, respectively. However, how these molecules regulate SSC self-renewal machinery remains unknown.

Reactive oxygen species (ROS), essential regulators of cell metabolism, are generated in virtually all cell types (D'Autréaux and Toledano, 2007). ROS are generated either by the mitochondrial electron transport chain or NADPH oxidases (Nox) (Kamata, 2009; Katsuyama et al., 2012). Unlike mitochondria oxidoreductases, Nox catalyzes the generation of O_2^- by single electron transfer from NADPH to O_2 . ROS are thought to act as a second messenger in several self-renewing tissues to regulate cellular activities. For example, hematopoietic stem cells (HSCs) generate ROS upon cytokine stimulation (Piccoli et al., 2006), and increased ROS production was observed in HSCs of aged animals (Ito et al., 2006). Excessive production of ROS in HSCs induces apoptosis via activation of the p38 MAPK-p16 pathway (Ito et al., 2006). However, conflicting reports exist on the effect of H_2O_2 supplementation on neural stem cells (NSCs). In one study, ROS generated by Nox2 in NSCs activate the

AKT pathway and induce proliferation (Le Belle et al., 2011), while other studies showed the inhibitory effect of ROS on NSC proliferation (Kim et al., 2009; Chuikov et al., 2010). ROS levels are involved in regulating proliferation, differentiation and genomic stability of embryonic stem cells (Li et al., 2010). In addition to normal stem cells, recent studies have also shown that cancer stem cells exhibit enhanced protection against ROS (Diehn et al., 2009). These results suggest that ROS are involved in both proliferation and differentiation of different stem cells in a context-dependent manner.

In spermatogenesis, oxidative stress has been generally associated with male infertility. Males with deficient NRF2, which regulates basal and inducible enzymes important for protection against ROS, have disruptive spermatogenesis in an age-dependent manner (Nakamura et al., 2010). Moreover, excessive ROS production in the cryptorchid condition induces DNA damage in spermatogenic cells, and spermatogonia in SOD1 KO mice showed poor resistance to heat stress (Ishii et al., 2005). A more recent study also showed that in ATM KO mice, cessation of spermatogenesis was accompanied by increased ROS in spermatogonia and Bax-dependent premeiotic germ cell loss (Takubo et al., 2008). Similar ROS accumulation by ATM deficiency was also observed in HSCs and compromised HSC activity (Ito et al., 2004), but, unlike bone marrow cells, ROS-mediated increase in p16 expression was not observed in ATM KO mouse testes. Moreover, depletion of ROS levels by drug treatment, which prevents HSCs from undergoing apoptosis, does not rescue spermatogenic defects (Takubo et al., 2006). Instead, p21 suppression partially restored the transplantation ability of ATM KO spermatogonia (Takubo et al.,

2008). Also, EpCAM⁺ spermatogonia, which are relatively enriched in committed spermatogonia (Kanatsu-Shinohara et al., 2011), express lower levels of cyclin D1 and D2, but not D3. Therefore, increased ROS levels were suggested to impair spermatogonia in a distinct manner from that observed in HSCs and decrease their proliferation by downregulating cyclins.

Contrary to these observations that show negative effects of ROS on spermatogonia, we hypothesized that ROS are beneficial for SSC self-renewal by acting downstream of Ras signaling because we observed that ROS were generated not only in Ras-GS cells but also in wild-type (WT)-GS cells upon self-renewal factor stimulation. The contribution of ROS to SSC self-renewal was analyzed by regulating ROS levels in vitro and analyzing SSC activities after ROS depletion in vivo by germ cell transplantation.

Results

Involvement of ROS in GS cell proliferation

In our search to identify molecules involved in GS cell proliferation, we found that addition of α -lipoic acid (LA), a ROS scavenger, abrogates proliferation of GS cells from C57BL6/Tg14(act-EGFP-OsbY01 (designated green) mice (Figure 1A). After 6 days, LA-treated cell recovery decreased in a dose-dependent manner (Figure 1B). ROS inhibition also abrogated proliferation of Ras-GS cells that self-renew without exogenous cytokines (Lee et al., 2009), suggesting that ROS act downstream of Ras signaling. Because Nox family is involved in ROS generation in many tissues,

we examined whether the Nox inhibitor diphenyl iodonium (DPI) suppresses their growth. We also tested another ROS scavenger and Nox inhibitor, apocynin (Figures 1A and B). After 6 days, < 10% of the input cells could be recovered after adding apocynin or DPI, suggesting that ROS generated by Nox genes are responsible for driving GS cell proliferation. Similar results were obtained in Ras-GS cells (Figure 1B).

Because only 1-2% of GS cells exhibit SSC activity and colonize seminiferous tubules of infertile recipient mice (Kanatsu-Shinohara et al., 2005), we performed transplantation experiments using congenitally infertile WBB6F1-W/W^v (W/W^v) mice to quantify SSC number after ROS depletion (Brinster and Zimmermann, 1994). In the first set of experiments using LA, we plated GS cells with LA and a portion of cells were transplanted at the culture initiation, and again after 6 days to evaluate the increase in SSC number. The number of colonies in recipient testes, which reflects SSC concentration in the transplanted cell suspension, generated by LA-treated cells and controls was comparable after 6 days (Figures 1C and D). However, the relative increase in total SSC number during 6 days (SSC concentration at 6 days × cell recovery/SSC concentration at culture initiation) was significantly diminished by LA (Figure 1E). In the second set of experiments using apocynin and DPI, the number of colonies in seminiferous tubules, as well as total SSC number in culture, was also significantly reduced. These results suggested that ROS production by Nox is important for SSC self-renewal.

Flow cytometric analyses using 2'-7'-dichlorodihydrofluorescein diacetate

(H₂DCFDA) showed that GS cells from B6-TgR(ROSA26)26Sor (ROSA26) mice (designated ROSA), which express LacZ in all germ cells, generate ROS upon addition of self-renewal factors 4 days after cytokine deprivation (Figure 1F). Although both FGF2 and GDNF increase ROS levels, we noted stronger ROS induction by FGF2, and a combination of FGF2 and GDNF showed a synergistic effect. ROS generation was inhibited by LA, as well as DPI and apocynin (Figure 1G), further suggesting the involvement of Nox genes in ROS generation. As expected from ROS inhibitor studies (Figure 1B), Ras-GS cells cultured without cytokines exhibited ROS production comparable to that observed for WT-GS cells cultured with cytokines (Figure 1H).

To determine the mechanism of ROS generation, GS cells were stimulated with cytokines in the presence of LY294002 or PD0325901, inhibitors of PI3K or MAPK pathways, respectively. We and others have previously shown that these pathways, which operate under Ras, contribute to GS cell proliferation (Lee et al., 2007; Oatley et al., 2007; Ishii et al., 2012). Inhibition of either pathway effectively suppressed cytokine-induced ROS generation (Figure 1I).

Increased GS cell proliferation by ROS elevation

Because hydrogen peroxide (H₂O₂) rather than superoxide has been implicated as a second messenger (Arnold et al., 2001), we examined whether increased H₂O₂ levels can influence GS cell proliferation. We supplemented GS cell cultures with various concentrations of H₂O₂, and measured cell recovery. Although no significant effects were observed at < 30 μ M, addition of > 100 μ M H₂O₂ inhibited

proliferation (Figures 2A and B). The inhibitory effect of H₂O₂ was accompanied by oxidative damage to DNA because increased γ H2AX staining was observed after H₂O₂ supplementation (Figures 2C and D). We also noted that oxidative stress decreased manganese superoxide dismutase (SOD2) levels, which detoxifies H₂O₂ (Figure 2E). However, we found that a physiological level of H₂O₂ (30 μ M) promotes proliferation when GS cells were continuously exposed to H₂O₂ for greater than 3 weeks (Figures 2B and F). The enhanced growth may be due in part to changes in adhesion properties of GS cells because we observed increased attachment of H₂O₂-treated GS cells to laminin-coated plates after overnight incubation (Figure 2G). Consistent with this observation, H₂O₂ supplementation increased expression of α 6-integrin, which comprises a laminin receptor. In contrast, expression of β 1-integrin and other spermatogonia markers, such as EpCAM and CD9, did not change upon addition of H₂O₂ (Figure 2H).

Cyclin overexpression enhances GS cell proliferation and impacts SSC colonization (Lee et al., 2009). Therefore, we examined the impact of H₂O₂ on cyclin D expression. Real-time polymerase chain reaction (PCR) analyses showed increased expression of cyclin D2 (Figure 2I), which is upregulated as a result of Ras activation in GS cells (Lee et al., 2009). We also found decreased cyclin D3 expression in H₂O₂-treated cells. However, no significant differences were observed in cyclin D1 expression. These results suggest that H₂O₂ may act by augmenting Ras-cyclin D2 pathway.

Increased SSC self-renewal and normal fertility of H₂O₂-treated GS cells

Because increased ROS production confers oxidative damage and influences cell differentiation, we performed germ cell transplantation to evaluate its effect on SSCs (Figure 3A). Green GS cells were cultured with 30 μ M H₂O₂ and transplanted into the seminiferous tubules of W/W^v mice at three time points during culture. Analyses showed that total cell number increased by 5.6×10^3 - and 1.0×10^3 -fold during the experimental period of 33 days, for H₂O₂-treated and control cultures, respectively. On the other hand, the concentration of SSC in culture was comparable between the two samples (Figures 3B and C). Therefore, the results indicate that H₂O₂ increased the total number of SSCs (SSC concentration at 33 days \times total cell increase/SSC concentration at culture initiation) by ~5.6-fold compared with untreated control cells. The doubling times of the SSCs were 2.6 and 3.3 days for the H₂O₂-treated and control SSCs, respectively.

Histological analyses of the recipient mice showed that H₂O₂-treated GS cells reinitiated normal appearing spermatogenesis (Figure 3D). To test whether the cells retain fertility, we sacrificed a recipient mouse that had been transplanted with GS cells (cultured for 52 days in the presence of H₂O₂) five months after transplantation. After cryopreserving the testes for 139 days at -80°C, spermatozoa were collected from freeze-thawed testes by repeated pipetting of the seminiferous tubules. Spermatozoa thus recovered were microinjected into oocytes, which were cultured for 24 h in vitro before transferring into uteri of pseudopregnant mice. A total of 27 embryos survived microinjection of freeze-thawed spermatozoa, and 11 two-cell embryos were

transferred to the uteri of pseudopregnant mothers. Three normal offspring were produced, all of which expressed the donor cell marker, enhanced green fluorescent protein (EGFP; Figure 3E). This birth rate (27%) was within the range of our previous experiments using normal spermatozoa from freeze-thawed testes (14-32%) (Ogonuki et al., 2006). These results indicate that GS cells treated with H₂O₂ have increased self-renewal potential and normal fertility.

Nox knockdown reduces GS cell proliferation and ROS generation

We next sought to identify which Nox molecule is responsible. Reverse transcription (RT)–PCR analyses showed that GS cells express Nox1, Nox3 and Nox4 (Figure 4A). Although Nox4 is constitutively and ubiquitously expressed, Nox1 and Nox3 are induced by cytokines (Kamata, 2009). Indeed, real-time PCR analyses showed that the expression of Nox1, but not Nox4, increases upon cytokine stimulation (Figure 4B). To test which Nox molecule is responsible for maintaining GS cell proliferation, we performed gene knockdown experiments using lentivirus vectors expressing short hairpin RNA (shRNA) against each Nox molecule. WT- and Ras-GS cells from green mice were infected with lentivirus vectors and cultured for 6 days after trypsinization. Real-time PCR analyses showed successful downregulation of target mRNAs (Figure 4C). After 6 days, inhibition of Nox1 expression significantly reduced WT-GS cell proliferation (Figure 4D), which was successfully rescued by adding H₂O₂ at the time of lentivirus infection (Figure 4E). Ras-GS cell proliferation was reduced by transfection of shRNA against Nox1 and Nox4 (Figure 4D). Inhibition

of Nox3 did not influence proliferation of WT- and Ras-GS cells. These results suggest that Nox1 is primarily responsible for maintaining GS cell proliferation by generating ROS.

Suppression of spermatogonia proliferation in vivo by LA or apocynin

The results of in vitro experiments showed the important role of ROS production in SSC self-renewal. To examine whether ROS is critical in vivo, we performed two sets of experiments. In the first set of experiments, we directly altered the ROS level in vivo by intraperitoneally injecting LA or apocynin into WT mice for 7 and 21 days, respectively. We used a magnetic bead selection procedure and confirmed decreased ROS levels by flow cytometry (Figure 5A). LA and apocynin treatments decreased ROS levels in both E-cadherin- and EpCAM-selected cells, while only apocynin treatment could decrease ROS levels in c-kit-selected cells, which could be due to the relatively short period for LA administration. Double immunohistochemistry showed decreased numbers of differentiating spermatogonia, as detected by c-kit expression, in both apocynin- and LA-treated animals (Figures 5B-E). Although cells expressing EpCAM, a marker for undifferentiated and differentiating spermatogonia, were also reduced by LA, they were not significantly affected by apocynin. Neither drug influenced the number of cells expressing E-cadherin, a marker for undifferentiated spermatogonia. Nevertheless, we observed that an increased proportion of E-cadherin⁺ cells expressed Ki67 in apocynin-treated animals. These results suggest that decreased ROS levels reduce the number of differentiating

spermatogonia and disturb the division of undifferentiated spermatogonia.

We quantified the influence of ROS depletion on SSCs by germ cell transplantation (Figure 5F). We administered LA or apocynin to green mice for 7 or 21 days, respectively, and single cell suspension of the testis cells was recovered for transplantation. Although LA administration reduced testicular weight at the end of treatment (Figure 5G), apocynin-treated testes were significantly smaller and fewer cells were obtained after enzymatic digestion (Figure 5H). The number of colonies generated by LA-treated and control donor cells was comparable (Figure 5I and J), but the total SSC number per testis (cell recovery \times colony number) was significantly reduced (Figure 5K). Similar results were obtained for apocynin. These results suggest that reduced ROS levels in vivo interfere with SSC self-renewal and decrease the total SSC pool size.

Spermatogonia proliferation is suppressed in Nox1 KO mice

In the second set of experiments, we used Nox1 KO mice to determine whether this gene is involved in SSC self-renewal in vivo (Matsuno et al., 2005). Nox1 KO male and female mice appear normal and are fertile. Although the seminiferous tubules contained all stages of spermatogenic cells and appeared normal (Figure 6A), double immunohistochemistry of Nox1 KO testis showed an overall reduction of Ki67⁺ cells as well as a decrease in EpCAM⁺ and c-kit⁺ cells (Figures 6B and C). This was accompanied by increased E-cadherin⁺ cells and increased Ki67 expression in E-cadherin⁺ and c-kit⁺ cells. To further characterize undifferentiated spermatogonia

fraction, we took advantage of PLZF and GFR α 1 antigens. The former is expressed in A_{single} (A_s), A_{paired} (A_{pr}), and A_{aligned} spermatogonia, while the latter is expressed in A_s and A_{pr} spermatogonia (Nakagawa et al., 2010). Although no differences were found in the frequency of Ki67⁺ cells, the total numbers of PLZF⁺ and GFR α 1⁺ cells were significantly decreased in Nox1 KO mice (Figures 6D and E). These results suggested that SSCs or their close descendents are decreased in Nox1 KO mice.

To assess the impact of Nox1 deficiency on SSCs, we transplanted Nox1 KO and WT testis cells into W/W^v mice in the first set of experiments. When we analyzed the recipient mice 3 months after transplantation, we noted a significant decrease in the colonization level of recipient testes that had received Nox1 KO testis cells, as assessed by testicular weight and the number of tubules with spermatogenesis in histological sections (Figures 6F-H). Because these results suggested decreased SSC numbers in Nox1 KO testis, we quantified SSCs by colony counts in the second set of experiments (Figure 6I). For this purpose, male Nox1 KO mice were mated with female green mice to introduce a donor cell marker for transplantation. No difference in the testis weight and cell recovery was observed between Nox1 KO and WT mice (Figures 6J and K). Analyses of the EGFP⁺ colonies 2 months after transplantation showed no significant differences in the number of colonies and total SSC numbers per testis (Figures 6L-N). Although these results suggested that the number of SSCs did not change upon Nox1 deficiency, poor colonization levels by histological sections in the first set of experiments suggested that Nox1 deficiency does not change the number of SSCs that seed in the recipients, but decreases the subsequent self-renewal in the

transplanted colonies.

To test this hypothesis, we performed serial transplantation experiments. The primary recipient testes were dissociated and a portion of the recovered cells was transplanted into secondary recipients to examine the effect of Nox1 deficiency during colony regeneration. Two months after transplantation, the number of EGFP⁺ colonies in the testes of the secondary recipients was analyzed under UV light. Assuming that one stem cell is responsible for the generation of each colony (Kanatsu-Shinohara et al., 2006) and that colonization efficiency is 10% (Nagano et al., 1999), the net increase in SSC number (the increase in the number of stem cells during the 2-months period in the primary recipient testis) was significantly smaller for Nox1 KO cells than control cells (Figure 6O). These results indicate that Nox1 deficiency reduces the rate of SSC self-renewal during colony regeneration in vivo.

Differential role of p38 MAPK and c-jun N-terminal kinase (JNK) in GS cell proliferation

Finally, we sought downstream molecules that mediate ROS signaling. Western blot analyses at 4 h after H₂O₂ supplementation showed that H₂O₂ does not influence AKT and MEK phosphorylation significantly (Figure 7A), which are implicated in ROS generation and SSC self-renewal (Lee et al., 2007; Nogueira et al., 2008; Ishii et al., 2012). However, we noted that phosphorylation of p38 MAPK and JNK, which are activated by ROS (Benhar et al., 2002), occurs upon H₂O₂ supplementation (Figure 7A). When GS cells started to show enhanced proliferation

after 3 weeks, p38 MAPK phosphorylation levels increased dramatically, suggesting that p38 MAPK plays an important role in enhancing H₂O₂-mediated proliferation during logarithmic growth phase. In contrast, although JNK2 phosphorylation did not change significantly, JNK1 phosphorylation became weaker during long-term culture.

Activation of p38 MAPK and JNK was also observed upon cytokine stimulation (Figures 7B and C). When GS cells were starved for 4 days and restimulated with cytokines, p38 MAPK and JNK were consistently phosphorylated, suggesting that they are involved in SSC self-renewal by extracellular signals. Phosphorylation of p38 MAPK and JNK was suppressed by ROS inhibitors (Figure 7D), suggesting that increased ROS levels by cytokines positively regulate their activities. Moreover, addition of the p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 abrogated proliferation of GS cells (Figures 7E and F). However, while SP600125 treatment did not significantly influence Nox gene expression, SB203580 treatment downregulated Nox1 expression (Figure 7G). These results suggested that Nox1 is regulated positively by p38 MAPK.

Using germ cell transplantation assays, we examined the effects of these inhibitors on SSCs. We transplanted the cells at the beginning and end of culture to measure the increase in SSC number. Although we failed to find significant differences in the number of colonies generated by SB203580-treated and control cells after 6 days of culture, the total cell number was significantly reduced by this treatment. Consequently, increase in SSC number (SSC concentration at 6 days × cell recovery/SSC concentration at culture initiation) was significantly decreased by

SB203580 treatment (Figures 7H-J). SP600125 treatment was more effective than SB203580, because we found significant decreases in the number of colonies, as well as total SSC number, upon SP600125 treatment (Figures 7H-J). Together, these results suggest that p38 MAPK and JNK contribute to GS cell proliferation in response to ROS generation by self-renewal factors.

Discussion

In this study, we found that ROS positively regulate SSC self-renewal. Depletion of ROS by LA, apocynin and DPI suppressed proliferation of WT- and Ras-GS cells. However, moderately increased ROS in GS cells by H₂O₂ supplementation significantly enhanced proliferation, although growth was suppressed at nonphysiological high concentrations. Such high ROS levels might have caused abnormalities in ATM KO testis, which were irreversible even upon ROS normalization (Takubo et al., 2006). The increased ROS production was mediated, in part, by Nox1, whose expression was upregulated upon cytokine stimulation. This notion was supported by experiments using Nox inhibitors and shRNA against Nox1, which suppressed GS cell growth. Although increased GS cell proliferation by ROS may be due to active proliferation of committed spermatogonia that have shorter cell cycles, transplantation experiments confirmed that increased ROS levels enhance GS cell proliferation without influencing SSC concentration and differentiating potential. These results suggest that ROS are necessary and sufficient for SSC self-renewal in vitro.

Using *in vitro* analyses, we found that PD0325901 and LY294002 suppressed ROS generation. Both MEK and AKT pathways have been implicated in ROS generation via Nox1 expression (Katsuyama et al., 2012). FGF2 and GDNF activate MEK and AKT pathways in spermatogonia (Meng et al., 2001; Goriely et al., 2009), but much remains unknown about their roles in SSC self-renewal. We recently showed that FGF2 and GDNF activate different sets of genes in GS cells and that FGF2 activates MEK more strongly than GDNF 30 min after cytokine stimulation (Ishii et al., 2012). MEK upregulates *Etv5*, which is essential for SSC self-renewal and also regulates ROS (Chen et al., 2005; Monge et al., 2009). Importantly, when GS cells overexpressing MEK or *Etv5* were cultured only with GDNF, they proliferated without losing SSC potential for long-term (Ishii et al., 2012). Although their growth was significantly slower than cells cultured with FGF2 and GDNF, this suggested that additional molecules, such as AKT, contribute to GS cell proliferation. Indeed, GS cells overexpressing AKT proliferated without GDNF when supplemented with FGF2 (Lee et al., 2007), and AKT promotes ROS production in several cell types by suppressing Forkhead box O (FOXO) transcription factors. In fact, mouse embryonic fibroblasts (MEFs) without AKT showed significantly reduced ROS due to decreased SOD2 and catalase expression, both of which are FOXO targets (Nogueira et al., 2008). Consistent with these data, a recent study showed that the PI3K-AKT pathway regulates FOXO1 stability in spermatogonia, and that loss of FOXO1 causes spermatogonia depletion, although ROS levels were not examined in these studies (Goertz et al., 2011). In this context, the suppression of ROS generation by PD0325901

and LY294002 raised the possibility that both AKT and MEK pathways regulate ROS levels to promote SSC self-renewal.

The importance of ROS in SSC self-renewal in vitro was assessed in vivo by depleting ROS in WT mice with chemical inhibitors. ROS depletion showed a profound effect in suppressing the proliferation of committed progenitor spermatogonia in both LA- and apocynin-treated mice. Although changes in undifferentiated spermatogonia compartment (as indicated by increased proliferation of E-cadherin⁺ undifferentiated spermatogonia) was only observed after apocynin administration, this could be due to the longer experimental period for apocynin and the fact that apocynin is more specific to Nox enzymes than LA. We also speculate that increased proliferation of E-cadherin⁺ cells was triggered by relative decrease in differentiating spermatogonia to compensate for the loss. Despite the relatively small effects on the number of E-cadherin⁺ undifferentiated spermatogonia, transplantation experiments confirmed significant loss in SSC activity by both inhibitors, which suggested the importance of ROS regulation in the maintenance of SSC activity.

Although Nox1 KO mice exhibited normal spermatogenesis, immunohistochemical analyses showed abnormalities in undifferentiated spermatogonia, including reduced numbers of GFR α 1⁺ and PLZF⁺ cells. However, the number of SSCs did not show significant difference between Nox1 KO and WT mice by transplantation assay. Nevertheless, because the seminiferous tubules of recipient animals with Nox1 KO donor cells showed poor colonization, we carried out serial transplantation and found limited SSC expansion in these recipient animals. In contrast

to in vitro cultures in which SSCs proliferate logarithmically, SSCs do not increase their number in the testis during normal spermatogenesis. This is probably why the effect of Nox1 deficiency on SSCs was not evident under steady conditions. Because differentiating spermatogonia were also decreased in Nox1 KO mice by immunohistochemistry, our results suggest that ROS are involved in proliferation of both SSCs and progenitors in vivo and their impact on SSCs becomes more dramatic when they are stimulated to increase their number during regeneration. Higher concentrations of GDNF in germ cell-depleted testes may have contributed to increased ROS generation and Nox dependency in SSCs (Tadokoro et al., 2002). Although the impact of Nox1 deficiency during normal spermatogenesis was limited compared to LA or apocynin, it is likely because this gene is induced by cytokines, and other constitutively expressed ROS generators, such as Nox4, may be more important during the steady state.

In our attempt to understand the growth promoting action of H₂O₂, we found that p38 MAPK and JNK are involved in SSC self-renewal. These kinases are known as stress kinases but often are involved in cell proliferation and development under physiological conditions (Benhar et al., 2002). Although inhibitor experiments showed that both are involved in SSC self-renewal by cytokine stimulation, Western blot analyses suggested a unique role for p38 MAPK. JNK and p38 MAPK phosphorylation occurred as early as 4 h after H₂O₂ stimulation, but p38 MAPK phosphorylation became stronger at later time points when cells are growing actively. These results suggested that unlike JNK, p38 MAPK responds relatively slowly to

ROS increase and is responsible for H₂O₂-mediated hyperproliferation. Consistent with this idea, several studies reported that p38 MAPK and JNK act differently according to the type of tissues (Benhar et al., 2002). Therefore, whereas activation of these kinases was suppressed similarly by ROS inhibition, they appear to contribute to SSC self-renewal in distinct manners. MAPK activation is regulated in a sophisticated manner by involving MAPK phosphatases, creating a feedback loop for regulation. Because H₂O₂ reversibly inactivates such phosphatases and increases the intensity of the ROS signal (Seth and Rudolf, 2006), p38 MAPK may be more susceptible to MAPK phosphatases than JNK and thus more sufficiently enhances proliferation of GS cells by ROS. Downregulation of Nox1 expression by p38 MAPK inhibition also suggests that ROS generation creates another positive feedback loop to amplify the original cytokine signal. Future analyses to elucidate the mechanism of p38 MAPK action as well as to identify its direct downstream targets are important next steps to understand the molecular machinery of SSC self-renewal.

Although ROS were thought to be hazardous to germ cells, this study provides evidence that they are indispensable for SSC self-renewal. While excessive ROS are apparently detrimental to SSCs by DNA damage, moderate ROS levels are necessary for self-renewal, suggesting that ROS levels need to be tightly controlled in these cells. Moreover, promotion of SSC self-renewal by p38 MAPK was unexpected, given that its suppression enhances self-renewal division of other stem cells, including HSCs, NSCs and intestinal stem cells (Ito et al., 2006; Sato et al., 2008; Sato et al., 2011). This suggests that ROS regulation in SSCs is distinct from that of stem cells of

other self-renewing tissues. In addition to intracellular regulation, ROS may be regulated by niche, a specialized microenvironment for stem cells. Because H_2O_2 is membrane permeable and can influence neighboring cells, one potential role of niche may be to regulate ROS levels. Indeed, studies on HSCs and NSCs showed that they reside in a hypoxic niche (Mohyeldin et al., 2010). In contrast, the location of the SSC niche has been controversial (Oatley and Brinster, 2012), and the current result raises question about whether the SSC niche plays a similar role. Therefore, clarifying the molecules and cell types that regulate ROS levels in SSCs will provide a new perspective in understanding the mechanism of SSC self-renewal and regulation by the microenvironment.

Experimental procedures

Cell culture

GS cells were derived from green or ROSA mice that were bred to a DBA/2 background for more than seven generations (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara et al., 2011). GS cells from green mice were used in transfection and transplantation experiments, whereas those from ROSA26 mice were used to measure ROS levels. Culture media were based on StemPro-34 SFM (Invitrogen, Carlsbad, CA) as previously described (Kanatsu-Shinohara et al., 2003). Growth factors used were 10 ng/ml human FGF2 and 15 ng/ml recombinant rat GDNF (both from Peprotech, London, UK). LA (2 mM), DPI (1 μ M; both from Sigma, St. Lois, MO), apocynin (1 mM; Tokyo Chemical Industry, Tokyo, Japan), SP600125 (40 μ M),

and SB203580 (30 μ M; both from Selleck chemicals, Houston, TX) were added to the cultures at the time of plating to examine their effects on cell proliferation. H₂O₂ was added at the indicated concentrations.

For laminin adhesion assays, green GS cells were plated on laminin-coated dishes (20 μ g/ml; BD Biosciences, Franklin Lakes, NJ) at a density of 3×10^5 cells/9.4 cm². After overnight incubation, the plates were washed twice with phosphate-buffered saline (PBS), and adherent cells were recovered by incubation for 5 min in 0.25% trypsin/1 mM ethylenediaminetetraacetic acid.

Statistical analyses

The results are presented as the mean \pm SEM. Independent samples with equal variance were analyzed using Student's *t*-test. Total SSC number per testis was determined by multiplying total cell recovery by SSC concentration, as determined by germ cell transplantation. For analyses of Nox1 KO testis serial transplantation, we developed a longitudinal model using PROC MIXED on SAS version 9.3 (SAS Institute, Cary, NC). In the model, we treated the generation of transplantation as time, and obtained the p-values of interaction between the presence of Nox1 and time in the model with Nox1, time, and interaction for cell counts.

Supplemental data

Supplemental data, including three tables and full methods (Supplemental Experimental Procedures) can be found with this article online.

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Figure legends

Figure 1. ROS generation in GS cells. (A) Appearance of GS cells after addition of ROS inhibitors. Cells were cultured for 6 days with the indicated inhibitors. (B) Suppression of WT- and Ras-GS cell proliferation by ROS inhibitors. Cells were cultured for 6 days with the indicated inhibitors ($n = 5-10$ for WT-GS cells, $n = 6$ for Ras-GS cells). Results of two experiments. (C) Macroscopic appearance of recipient testes that were transplanted with GS cells cultured for 6 days with the indicated inhibitors. Green tubules indicate spermatogenesis from donor SSCs. (D, E) Colony count (D) and total increase in SSC number during 6 days (E; $n = 17$ for LA, $n = 13$ for apocynin, and $n = 12$ for DPI). Results of three experiments. (F) Flow cytometric analyses of intracellular ROS generation after cytokine supplementation. Four days after cytokine deprivation, cells were stimulated with the indicated cytokines and analyzed 4 h after the treatment. (G) Suppression of ROS generation by ROS inhibitors. Four days after cytokine deprivation, cells were treated with the indicated inhibitors for 1 h before stimulation by FGF2 and GDNF. Cells were analyzed 4 h after treatment. (H) Increased ROS generation in Ras-GS cells. Ras-GS cells that had been cultured without cytokines were compared with WT-GS cells cultured with FGF2 and GDNF. (I) Suppression of ROS generation by MEK and PI3K inhibitors. Four days after cytokine deprivation, cells were treated with the indicated inhibitors for 1 h before stimulation by FGF2 and GDNF. Cells were analyzed 4 h after treatment. Bar = 100 μm (A), 1 mm (C). Error bars = SEM.

Figure 2. H₂O₂ increases proliferation of GS cells. (A) Appearance of GS cells after the addition of H₂O₂ for 6 days. (B) Effect of H₂O₂ on GS cell recovery (n = 6). Results of two experiments. High concentrations of H₂O₂ inhibited proliferation of GS cells. Although no significant effects were observed at low H₂O₂ concentrations at 6 days, cells proliferated more actively after 3 weeks. (C) Immunocytochemistry of γ H2AX expression. GS cells showed increased signals when cells were cultured with 500 μ M H₂O₂ overnight. (D) Quantification of cells with γ H2AX staining. Cells expressing γ H2AX in five random fields were counted (n = 3376 for 30 μ M, n = 1384 for 500 μ M, and n = 3462 for control). (E) Western blot analyses of SOD2 expression after H₂O₂ stimulation. (F) Cumulative growth curve of GS cells cultured with 30 μ M H₂O₂. (G) Increased binding to laminin upon H₂O₂ supplementation. GS cells were incubated overnight on laminin-coated plates and recovered by trypsin digestion (n = 18). Results of two experiments. (H) Flow cytometric analyses of surface marker expression. Note the increased α 6-integrin expression. (I) Real-time PCR analysis of cyclin D expression in H₂O₂-treated cells (n = 9). Results of three experiments. Bar = 100 μ m (A, C). Error bars = SEM. See also Tables S1 and S2.

Figure 3. Functional analyses of cultured cells by germ cell transplantation. (A) Experimental strategy for production of offspring from GS cells. (B) Macroscopic appearance of recipient testes transplanted with H₂O₂-treated GS cells. Green tubules indicate spermatogenesis from donor SSCs. (C) Colony counts (n = 14). Results of

three experiments. (D) Normal spermatogenesis from H₂O₂-treated GS cells 5 months after transplantation. Spermatozoa used in microinsemination are indicated in the inset (arrow). (E) Offspring produced by microinsemination showing fluorescence under UV light. Bar = 1 mm (B), 100 μ m (D). Error bars = SEM.

Figure 4. Regulation of GS cell proliferation by Nox genes. (A) RT-PCR analyses of Nox gene expression. (B) Real-time PCR analyses of Nox1 and Nox4 mRNA expression (n = 3). Results of three experiments. (C) Real-time PCR analyses of Nox gene expression 6 days after transfection of shRNA vectors (n = 3). Results of three experiments. (D) Suppression of WT- and Ras-GS cell proliferation by Nox gene knockdown (n = 9). Cells were recovered 6 days after transfection. Results of three experiments. (E) Rescue of Nox1-induced proliferation suppression by H₂O₂ (n = 6). Cells were recovered 6 days after transfection. Results of two experiments. H₂O₂ was added at the time of transfection. Error bars = SEM. See also Table S1.

Figure 5. Suppression of spermatogonia proliferation in vivo upon ROS depletion. (A) Flow cytometric analyses of intracellular ROS generation after ROS depletion in vivo. Cells with indicated markers were recovered by magnetic cell sorting and analyzed for their ROS levels. (B, C) Immunohistochemical staining of spermatogonia marker and Ki67 expression in WT testes after LA (B) or apocynin (C) administration. (D, E) Quantification of cells with spermatogonia marker expression after LA (D) or apocynin (E) administration. Twenty tubules were counted. (F) Experimental strategy to quantify

SSCs after LA or apocynin administration. (G, H) Testis weight (G) and cell recovery (H) after treatment. Testes of LA (n = 6 for LA, n = 4 for control) or apocynin (n = 8 for apocynin, n = 8 for control)-treated mice were recovered following 7 and 21 days of treatment, respectively. (I) Macroscopic appearance of recipient testes transplanted with LA or apocynin-treated green mouse testis cells. Green tubules indicate spermatogenesis from donor SSCs. (J, K) Colony count (J) and total SSC number (K) in the donor testis after LA (n = 24) or apocynin (n = 10) administration. Results of four (LA) and two (apocynin) experiments. Bar = 20 μ m (B, C), 1 mm (I). Error bars = SEM. See also Tables S2 and S3.

Figure 6. Suppression of spermatogonia proliferation in Nox1 KO mice. (A) Normal appearing spermatogenesis in Nox1 KO testis. (B) Immunohistochemical staining of spermatogonia marker and Ki67 expression in Nox1 KO testis. (C) Quantification of cells with spermatogonia marker expression. Fifteen tubules were counted. (D) Immunohistochemical staining of undifferentiated spermatogonia marker and Ki67 expression in Nox1 KO testis. (E) Quantification of cells with undifferentiated spermatogonia marker expression. Thirty tubules were counted. (F) Histological appearance of recipient testes transplanted with testis cells from Nox1 KO mice. (G) Testis weight of recipient mice transplanted with Nox1 KO testis cells (n = 17 for Nox1 KO, n = 14 for control). Results of two experiments. (H) Tubules with spermatogenesis, defined as the presence of multiple layers of germ cells in the entire circumference of the tubules, were counted. The total numbers of tubules counted were

2429 and 2414, respectively, for Nox1 KO and control donor testis cells (n = 20 for Nox1 KO, n = 24 for control). Results of two experiments. (I) Experimental strategy for serial transplantation. (J, K) Weight (J) and cell recovery (K) of Nox1 KO donor mouse testes (n = 4). (L) Macroscopic appearance of the primary and secondary recipient testes following transplantation of Nox1 KO testis cells. Green tubules indicate spermatogenesis from donor SSCs. (M) Colony count in the primary recipients (n = 24). Results of four experiments. (N) Total SSC number in Nox1 KO testis. (O) Total increase of colony numbers (total regenerated colony number \times 10) – (primary colony number used for serial transplantation)(n = 16 for Nox1 KO; n = 15 for control; p = 0.04 for interaction between Nox1 and timing of transplantation). Bar = 100 μ m (A), 20 μ m (B), 10 μ m (D) 500 μ m (F), 1 mm (L). Error bars = SEM. See also for Tables S2 and S4.

Figure 7. Regulation of GS cell proliferation by p38 MAPK and JNK. (A) Western blot analyses of GS cells cultured with H₂O₂. (B, C) Western blot analyses of p38 MAPK (B) and JNK (C) phosphorylation after cytokine treatment. Cells were starved for 4 days, and the samples were collected 4 h after cytokine addition. (D) Western blot analyses of p38 MAPK and JNK phosphorylation after ROS depletion. Cells were cultured for 6 days with FGF2 and GDNF. Apocynin was added on the second day after plating, while LA and DPI were added on the fourth and fifth days, respectively. (E) Appearance of GS cells after addition of SB203580 or SP60012. Cells were cultured for 6 days with the indicated inhibitors. (F) Inhibition of GS cell proliferation

by SB203580 or SP60012. Cells were cultured for 6 days with the indicated inhibitors (n = 6). Results of two experiments. (G) RT-PCR analyses of Nox gene expression. Cells were cultured for 2 days with the indicated inhibitors. (H) Macroscopic appearance of recipient testes that received SB203580 or SP60012-treated GS cells. (I, J) Colony count (I) and total increase in SSC number (J) after SB203580 or SP60012 treatment (n = 13 for control and SB203580, n = 16 for control and SP60012). Results of two (SB203580) or three (SP60012) experiments. Bar = 100 μ m (E), 1 mm (H). Error bars = SEM. See also Tables S1 and S2.